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FINAL REPORT

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NUTRACEUTICAL POTENTIAL OF BRASSICA SPROUT EXTRACTS

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NUTRACEUTICAL POTENTIAL OF BRASSICA SPROUT EXTRACTS

FINAL REPORT: March, 2002

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SUMMARY

Project objectives: determine whether cruciferous sprouts high in glucoraphanin could prevent inflammatory cardiovascular changes and to identify crucifers high in glucoraphanin that could be readily grown in Saskatchewan. Hypertensive stroke prone rats fed dried broccoli sprouts (high in glucoraphanin) were less hypertensive and showed almost no inflammatory changes in blood vessels and other organs compared to the control diet animals. We demonstrated that hoary cress and field peppergrass have a high glucoraphanin content in the mature plant. We conclude that field peppergrass has the potential of being developed into an anti-inflammatory nutraceutical crop.

EXECUTIVE SUMMARY

The project had two objectives. One was to determine whether a diet of crucifers high in glucoraphanin (the precursor of sulforaphane) could prevent inflammatory changes in the cardiovascular system. Such inflammatory changes lead to atherosclerosis and atherosclerosis can result in heart attacks and strokes. The second objective was to identify crucifers that could be readily grown in Saskatchewan that were high in glucoraphanin.

RESULTS

Identification of Crucifers High in Glucoraphanin: The first thing we did was to develop our laboratory the HPLC analytical technique to measure glucosinolates. To verify that the HPLC peak we thought was glucoraphanin was glucoraphanin, we had mass spectrum analysis performed by L. Hogge of the College of Pharmacy & Nutrition. When we were confidant we could identify glucoraphanin we began to analyze crucifers for glucosinolate contents. More than 250 crucifer lines have been analyzed for glucosinolate profiles. Of pertinence to this report is that we identified a broccoli cultivar that contained high levels of glucoraphanin but few other glucosinolates at the sprout stage of development. With broccoli, there is as much glucoraphanin in the sprout as in the mature plant. We obtained 20 kg of seed of this cultivar and are using this broccoli cultivar for our feeding experiments. We identified two crucifers that had a high content of glucoraphanin in the mature plant. These were hoary cress (*Cardaria draba*), a noxious weed, and field peppergrass (*Lepidium campestre*). Both of these species also had high content of the anti-nutritive glucosinolate, glucosinalbin.

There appear two directions to go for the development of a nutraceutical product. One is to use broccoli sprouts; however, there is a patent on the medicinal use of crucifer sprouts and this direction may be difficult. Another direction to go is the use of mature plants, where there are no patents involved. Since hoary cress is a noxious weed, field pepperrgrass appears to be a promising plant to explore. There are two possible ways of developing field peppergrass as a nutraceutical. One is to develop extraction technologies that will selectively extract glucoraphanin. The other is to use classical mutagenesis to knock out the glucosinalbin.

An NSERC-funded student, Erin Powell, working under the supervision of Dr. Gordon Hill of Chemical Engineering, is examining the effect of different extraction procedures on

glucosinolate isolation. She has demonstrated that optimal extraction of glucosinolates is by using hot 20% ethanol with optimal pH being at pH 3. An extraction time of 20 minutes was as efficient as an extraction time of 120 minutes. However, glucosinalbin was extracted as efficiently as glucoraphanin.

Effect of a Diet High in Glucoraphanin on Inflammation in Blood Vessels and Other Organs

We have run dietary experiments on male and female spontaneously hypertensive stroke-prone rats. These rats develop hypertension, type 2 diabetes and inflammatory changes in blood vessels that resemble atherosclerosis, inflammatory changes in kidneys, liver, brain and spinal cord. These inflammatory changes resemble those seen in ageing human beings. Experiments were conducted on both male and female rats, with feeding trials being from the age of two months to six months. All the analyses have not yet been completed; in fact, some of the feeding trials are still going on. Below is a summary of the findings of the data so far collected and interpreted.

Effect on Blood Pressure: The rats on control diet develop hypertension to a mean blood pressure of 185/155 mm Hg. Blood pressure increases start at 2 months and reach maximal levels by about 5 months of age. Animals were placed on specific diets from 2 to 6 months of age. Feeding female animals with 200 mg dried broccoli sprouts containing 2.4 μmoles glucoraphanin resulted in blood pressures being 24 mm Hg lower than animals fed with control diets; this difference was statistically significant. With males, the blood pressures of the broccoli fed animals were 20 mm Hg lower than animals fed control diet. Feeding dried canola sprouts resulted in blood pressures that were only slightly lower, but significantly lower, than animals fed with control diets.

The stroke-prone rats develop high blood pressure for some of the same reasons as do people. One of the causes is damage to the endothelial lining of the blood vessels such that the endothelial cells do not produce adequate amounts of nitric oxide in response to stimuli such as acetylcholine. We examined the contractile properties of the aorta of the animals placed on the different diet. We found that in the control and canola diet fed female rats the endothelial lining of the aorta produced less nitric oxide in response to acetylcholine than the endothelial lining of

the broccoli fed animals. In other words, acetylcholine could make contracted aorta relax in broccoli-fed animals but it caused little relaxation in control and canola diet-fed animals. This is in keeping with the hypothesis that initiated these experiments. The hypothesis was that oxidative stress at the level of the blood vessels would cause hypertension and atherosclerosis-like inflammatory changes and that feeding animals with glucoraphanin would decrease oxidative stress and the associated problems. Our analysis of oxidative stress in blood vessels is not yet completed but the results so far indicate that a diet of broccoli sprouts in decreased oxidative stress.

We also demonstrated that glucoraphanin is metabolized sulforaphane when broccoli sprouts are allowed to freeze and then thawed and dried; this was expected. Feeding animals such dried sprouts (where the glucoraphanin was metabolized) had no effect on blood pressure.

Effect on Inflammatory Changes in Blood Vessels: One measure of inflammation is the infiltration of vessels by activated macrophages. Activated macrophages make a major contribution to the development of atherosclerosis lesions. We found that on control and canola diets there was infiltration of activated macrophages both in small vessels such as found in the coverings of the brain as well as large vessels such as the aorta. Very few activated macrophages could be found associated with the blood vessels of broccoli sprout fed animals.

Effect on Inflammatory Changes in Other Organs: Control diet fed animals had large numbers of activated macrophages in all organs examined to date, including kidneys, liver and brain. Few activated macrophages were seen in broccoli sprout fed animals.

Effect of a Diet High in Glucoraphanin on Oxidative Stress in Organs: The hypothesis underlying this research is that glucoraphanin is metabolized to sulforaphane, a compound that increases the anti-oxidant defense systems of organs. If this is the case, one would expect that organ glutathione would be increased. One would also expect that organ glutathione reductase and glutathione peroxidase activities would increase. We have begun analyses of the oxidative status of the various organs with the kidney data now completely collected. We have shown that glutathione levels in kidneys from broccoli fed animals is almost double that from control diet fed animals. We have also shown that there is a 50% increase in glutathione reductase activities

and glutathione peroxidase activities in the broccoli sprout fed animals compared to control diet fed animals.

CONCLUSIONS

This is the first study showing that a dietary intervention can decrease oxidative stress and associated inflammation. We have shown: 1) a diet high in glucoraphanin does decrease oxidative stress in organs, 2) the decreased oxidative stress was associated with decreased inflammation in a variety of organs, including the cardiovascular system, and 3) the decreased oxidative stress was also associated with a decrease in severity of hypertension. These findings fit well with the hypotheses that initiated this research.

Of the crucifers examined, *Lepidium campestre* (field peppergrass) seems to hold the most promise for the development of a nutraceutical crop in this province. The reason for this is that the mature form of the plant has high levels of glucoraphanin (~0.6% of dry weight).

IMPLICATIONS

Our data indicate that it is possible to develop a nutraceutical product that has powerful anti-inflammatory properties.

RECOMMENDATIONS

Research be conducted on: 1) developing extraction procedures for glucoraphanin, 2) developing a field peppergrass that continues to have a high glucoraphanin content but with low glucosinalbin content.

TECHNICAL REPORT

BACKGROUND

The theoretical background to this project is outlined in several published manuscripts that acknowledge ADF funding (Christman et al., 2000; Juurlink, 2001a; Juurlink, 2001b). The theory is that dietary phase 2 protein inducers will increase phase 2 protein gene expression with the consequence that oxidative stress and associated inflammation is decreased. There is good evidence that oxidative stress at the level of the smooth muscle cells in blood vessels can lead to hypertension (Wu and de Champlain, 1999). We have shown that smooth muscle cells of hypertensive rats experience much more oxidative stress than smooth muscle cells from normotensive rats ; furthermore, we have shown that sulforaphane, the phase 2 protein inducer that is a metabolite of the glucosinolate glucoraphanin, can reduce oxidative stress in these smooth muscle cells grown in culture (Wu and Juurlink, 2001).

Sulforaphane is the metabolite of glucoraphanin. Previous research by Talalay and colleagues demonstrated that sulforaphane is a potent phase 2 protein inducer and when taken via the diet either as the isothiocyanate sulforaphane or the precursor glucosinolate, glucoraphanin, dietary intake could inhibit the formation of chemically –induced cancers (Fahey et al., 1997). Talalay and colleagues also showed that broccoli sprouts, but not the mature broccoli plant, is a rich source of glucoraphanin, and have patented the use of crucifer sprouts for medicinal purposes.

The reason that induction of phase 2 proteins better enable cells to inhibit cancer formation is that phase 2 protein induction increases the ability of cells to scavenge strong oxidants that can cause DNA mutation. It occurred to me that more efficient scavenging of strong oxidants should also decrease inflammation since strong oxidants can activate inflammatory pathways (Christman et al., 2000; Juurlink, 2001b). This resulted in the hypothesis that increased dietary intake of phase 2 protein inducers should decrease many of the problems associated with ageing, such as hypertension, atherosclerosis, problems associated with type 2 diabetes and possibly even Alzheimer's disease.

PROJECT OBJECTIVES

The project had two objectives. One was to determine whether a diet of crucifers high in glucoraphanin (the precursor of sulforaphane) could prevent inflammatory changes in the cardiovascular system. Such inflammatory changes lead to atherosclerosis and atherosclerosis can result in heart attacks and strokes. The second objective was to identify crucifers that could be readily grown in Saskatchewan that were high in glucoraphanin.

PROJECT ACTIVITIES

1) We screened more than 250 crucifer lines for glucosinolate profiles.
2) We examined various storage conditions for the development of a nutraceutical product and are in the process of examining means of extracting glucoraphanin.
3) We tested animals for the effect of a diet containing 10 μmoles glucoraphanin/kg body weight

ANIMAL EXPERIMENTAL DATA

Animal experimentation is very labour intensive. One person is required almost full-time for ensuring that the animals are weighed weekly, fed their portion of sprouts daily and blood pressures taken once a week. In addition, one technician is kept busy half-time measuring glucosinolates in plant extracts and thiols in the tissues of the experimental animals. For the past 6-8 months a College of Medicine funded post-doctoral fellow, Dr. Hossein Noyan, has been examining for inflammatory changes in the brain, spinal cord, liver and kidney. In addition, a CIHR/Heart & Stroke Foundation funded post-doctoral fellow, Dr. Lingyun (Lily) Wu, has been examining blood vessel physiological responses and measuring anti-oxidant enzyme activities in tissue of the experimental animals. Since September, Ms Marina Facci, an M.Sc. student funded in part by the College of Medicine Graduate Scholarship fund has been examining for inflammatory changes in the blood of these animals. Over the past year the main focus of the project has been the animal experiments.

Blood Pressure

Blood pressure measurements have been taken from both female (Figure 1A) and male (Figure 1B) stroke-prone rats on various diets. The measurements of the first batches of animals have been taken and analyzed, the second batch of female and males are still being measured. Female and male animals on broccoli sprouts had a systolic blood pressures that were 20-24 mm Hg less ($P<0.001$) than animal on control diets. In the female batch of animals we also examined the effect of canola sprouts and demonstrated that canola sprout-fed animals had a 10 mm Hg lower systolic blood pressure ($P<0.05$). With the male batch of animals we examined the effect of freezing than thawing broccoli sprouts before drying; this caused the glucoraphanin to be metabolized to sulforaphane, a very reactive compound. Rats fed such frozen-thawed-dried broccoli sprouts had blood pressures that were indistinguishable from the animals fed control diets (Figure 1B). We conclude that conversion of glucoraphanin to sulforaphane caused the sulforaphane to be inactivated before the sprouts were fed to the animals.

Our overall conclusion from these experiments is that providing stroke-prone animals a diet high in glucoraphanin decreases the development of hypertension.

Blood Vessel Physiological Responses

Hypertension can be due, at least in part, by the inability of the endothelial lining of the blood vessel to respond to signals such as acetylcholine by synthesizing nitric oxide. This nitric oxide then diffuses to the adjacent smooth muscle cells causing relaxation of the smooth muscle cells, thereby decreasing blood pressure. To test the effect of the various diets on the relaxation abilities of the aorta, at 6 months, segments of the thoracic aorta were taken and placed into an instrument that can measure contraction and relaxation of the vessels. The aortae were contracted by placing phenylephrine in the bath. Acetylcholine was then administered and the ability of the vessels to relax was then quantified. Figure 2 shows the results observed for the first experiment with female rats. It is clear that aortae of the canola sprout-fed animals do not relax as well as the broccoli sprout-fed animals. A second batch of females that have been fed various diets from 2 months to 6 months of age will be examined before the middle of March.

Figure 3 gives the data on the response of male rats to control diets or diets containing broccoli sprouts containing either glucoraphanin or where the glucoraphanin has been converted to sulforaphane. Again, it is clear that a diet containing broccoli sprouts better enables the aortae to relax in response to acetylcholine when previously contracted in response to phenylephrine. Surprisingly, considering the blood pressure data, broccoli sprouts that were frozen, thawed and then dried (i.e., in sprouts where the glucoraphanin is converted to sulforaphane),

To determine whether such differences were due to differences in the ability of the smooth muscle cells to respond to nitric oxide or due to differing abilities of the endothelial cells to produce nitric oxide in response to acetylcholine, we examined the ability of the blood vessels to relax in response to a compound (SNP) that releases nitric oxide. Figure 4 gives the data for the female rats while Figure 5 gives the data for the male animals. With females, the smooth muscle cells from the animals on the canola diet were much more responsive to nitric oxide than the animals on the broccoli sprout diet. This was the case, although not as dramatic, when control diets were compared to broccoli diets in the males.

We interpret our data on the physiological responses of the aortae to indicate that with control or canola sprout-containing diets, the endothelial lining of the blood vessels produce little nitric oxide in response to normal physiological signals such as acetylcholine, whereas, this is not the case when animals are put on a diet containing glucoraphanin. Furthermore, this reduction in the nitric oxide signal in the animals fed control or canola-containing diets causes the smooth muscle cells to become hyper-responsive to nitric oxide.

Blood Vessel Inflammatory Changes

One measure of inflammation is the infiltration of vessels by activated macrophages. Activated macrophages make a major contribution to the development of atherosclerosis lesions. We found that on a control diet there was infiltration of activated macrophages both in small vessels such as found in the coverings of the brain (Figure 6) as well as large vessels such as the aorta (Figure 7). Very few activated macrophages could be found associated with the blood vessels of broccoli sprout fed animals.

Inflammatory Changes In Other Organs

Inflammatory changes as evidenced by the presence of activated macrophages using immunocytochemistry for the ED1 marker were also seen in the liver (Figure 8), kidney (Figure 9) and brain (Figure 10) of animals fed either broccoli or control diets. There was little evidence of inflammation in these organs from broccoli fed animals. We are currently examining for other markers of inflammation such as inducible nitric oxide synthase and nitrotyrosine residues.

Measurements Of The Anti-Oxidant Defense Systems

We have collected tissues to measure the anti-oxidant defense systems. To date we have completed the examination of the kidneys in male animals. As can be seen in Figure 11, Feeding broccoli sprouts to animals significantly increases kidney glutathione levels. Glutathione plays a critical role in many of the strong oxidant scavenging systems. Broccoli intake also significantly increases glutathione peroxidase activities (Figure 11) and glutathione reductase activities (Figure 12) but had no effect on glutathione S-transferase activities (Figure 12). The same

tendency for increasing the anti-oxidant defense systems seen with dried broccoli sprouts was also seen with the frozen-thawed-dried broccoli sprouts; however, the increase in the anti-oxidant defense systems was not as pronounced. We are currently examining other tissues and organs, including the aorta.

DISCUSSION OF RESULTS

This is the first study showing that a dietary intervention can decrease oxidative stress and associated inflammation. We have shown: 1) a diet high in glucoraphanin does decrease oxidative stress in organs, 2) the decreased oxidative stress was associated with decreased inflammation in a variety of organs, including the cardiovascular system, and 3) the decreased oxidative stress was also associated with a decrease in severity of hypertension. The data have been collected from both female and male animals and the findings are similar.

These findings fit well with the hypotheses that initiated this research. This hypothesis was that a diet high in a phase 2 protein inducer would decrease oxidative stress and associated inflammation. Our results show that a relatively modest change in diet can have marked effects of the cardiovascular system as well as other organs. This suggests that there would be a significant market for a nutraceutical product high in phase 2 protein inducer.

Of the crucifers examined, *Lepidium campestre* (field peppergrass) seems to hold the most promise for the development of a nutraceutical crop in this province. The reason for this is that the mature form of the plant has high levels of glucoraphanin (~0.6% of dry weight).

INDICATIONS FOR FUTURE DEVELOPMENT

Our data indicate that it is possible to develop a nutraceutical product that has powerful anti-inflammatory properties.

RECOMMENDATIONS

Research be conducted on: 1) developing extraction procedures for glucoraphanin, 2) developing a field peppergrass that continues to have a high glucoraphanin content but with low glucosinalbin content.

PERSONNEL

The following personnel have been paid partially or completely off the ADF grant:

Anita Quon – technician. Ms Quon when she took another job was replaced by Ms Arlene Drimmie.

Yi Qian Wang – technician, hired on a part-time basis.

Karen Coulman (summer of 1999), summer student was paid via aUSTEP/ grant supplemented by ADF funds
Joy Agnew (summer of 2000), summer student was paid via aUSTEP/ grant supplemented by ADF funds

Erin Powell (2001/2002) NSERC-funded M.Sc. student supplemented from ADF funds

Marina Facci (from September 2001) College of Medicine funded (50%) student supplemented from ADF funds

PROJECT DEVELOPED MATERIALS

To date, one abstract has acknowledged described our findings and ADF support. We are currently writing a manuscript on our animal findings to be submitted to Nature Medicine.

ACKNOWLEDGEMENTS

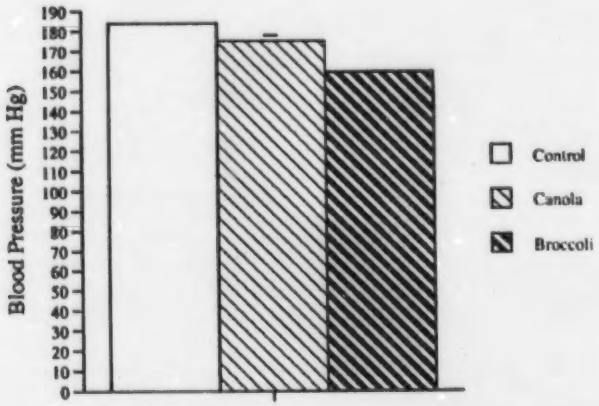
To date 3 review papers plus one abstract has acknowledged ADF support. In addition, presentations at the Saskatchewan Nutraceutical Network and PBI have acknowledged ADF funding. A seminar will be given to the Department of Plant Sciences on March 12 where ADF support will be acknowledged. In addition, we have begun to write manuscripts to describe the findings with the animal experiments where ADF support will be acknowledged.

EXPENSE STATEMENT

Yet to be completed but will be submitted shortly.

Figure 1A

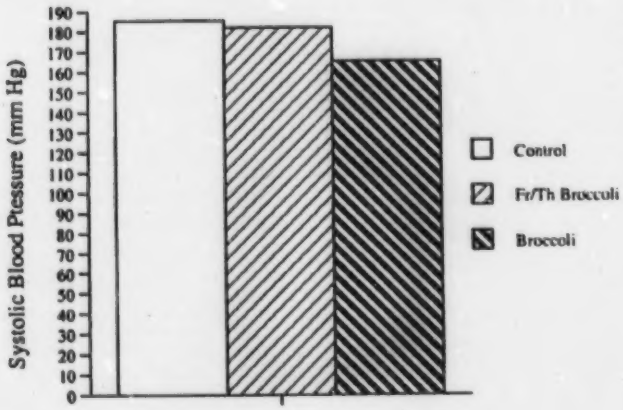
Systolic Blood Pressure in Female Stroke-Prone Rats



Data re means ± standard errors of the mean. Control n=8, canola n=6, broccoli n=5.

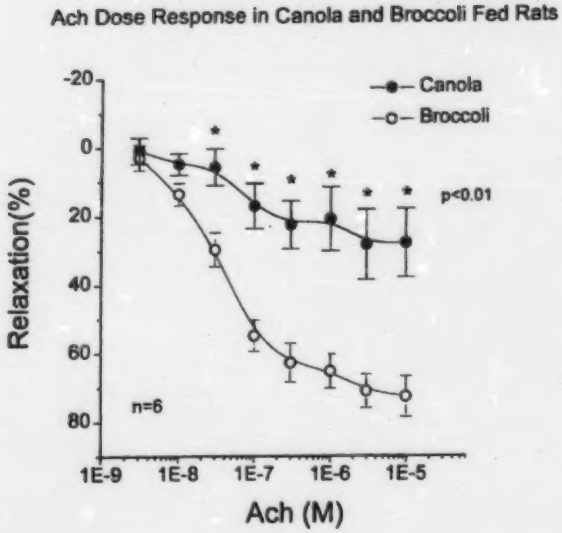
Figure 1B

Systolic Blood Pressure in Male Stroke-Prone Rats



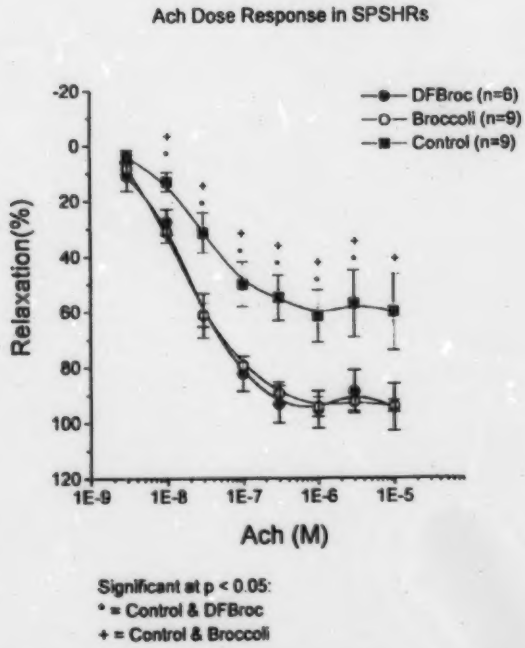
Data re means ± standard errors of the mean. Standard error bars are too small to be seen. Control n=7, frozen, thawed and then dried broccoli (Fr/Th) n=8, broccoli n=8.

Figure 2



There were 6 animals in each group.

Figure 3



DFBroc indicates broccoli sprouts that have been frozen, thawed and then dried, whereas Broccoli indicates sprouts that have been air-dried.

Figure 4

SNP Dose Response in Canola and Broccoli Fed Rats

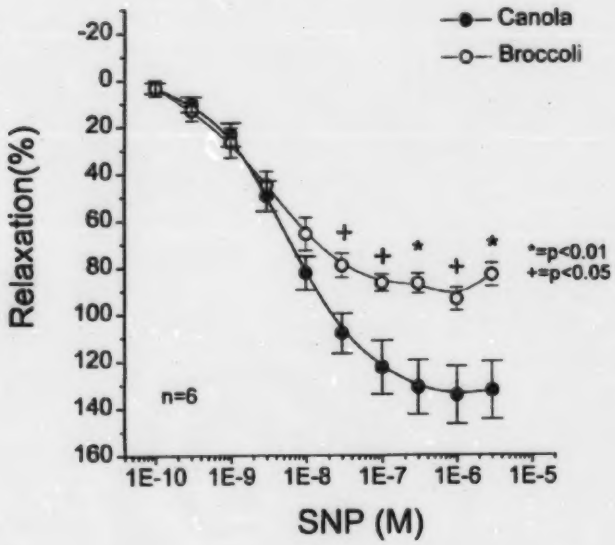
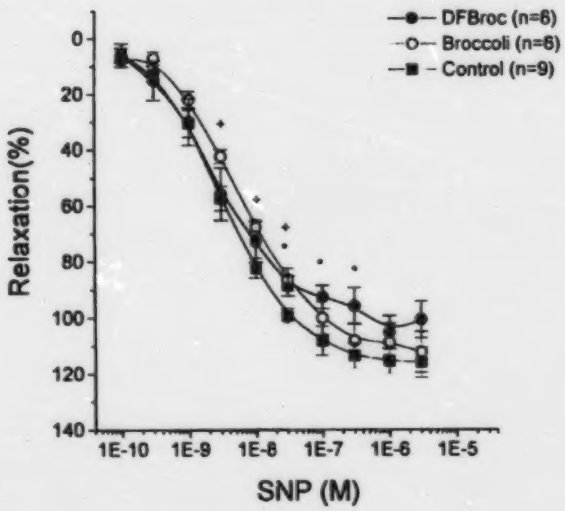


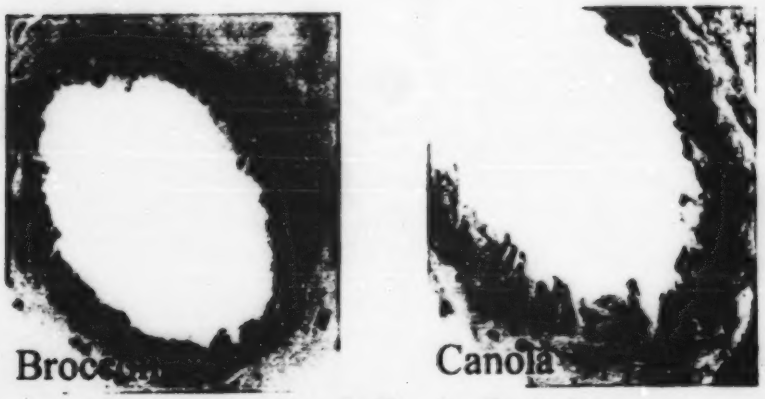
Figure 5

SNP Dose Response in SPSHRs

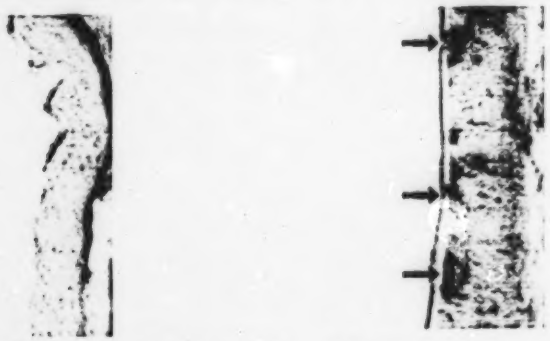


Significant at p < 0.05:
* = Control & DFBroc
+ = Control & Broccoli

Figure 6



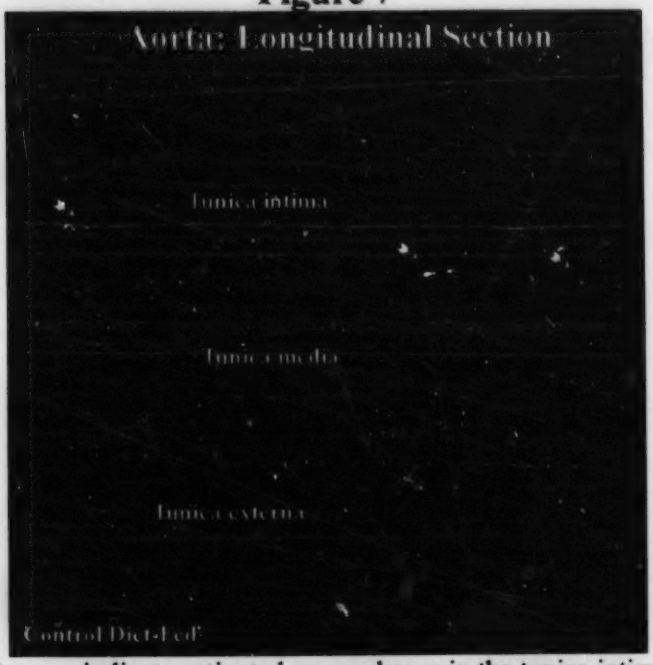
Hematoxylin & Eosin Staining



OX42 Staining For Macrophages

Meningeal blood vessels showing disruption of the intermediate layer (hematoxylin & eosin staining) in the canola fed animals. The canola fed animals also had macrophages infiltrating the meningeal vessels.

Figure 7



Arrows indicate activated macrophages in the tunica intima

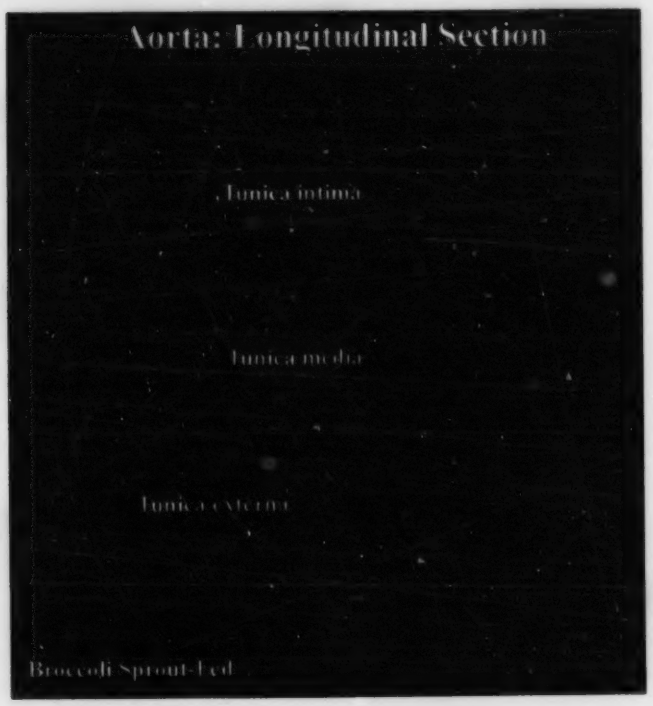
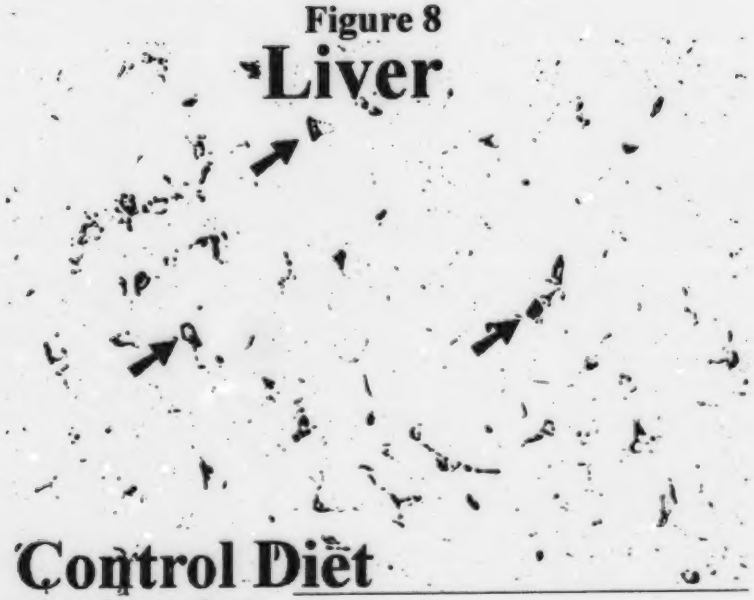
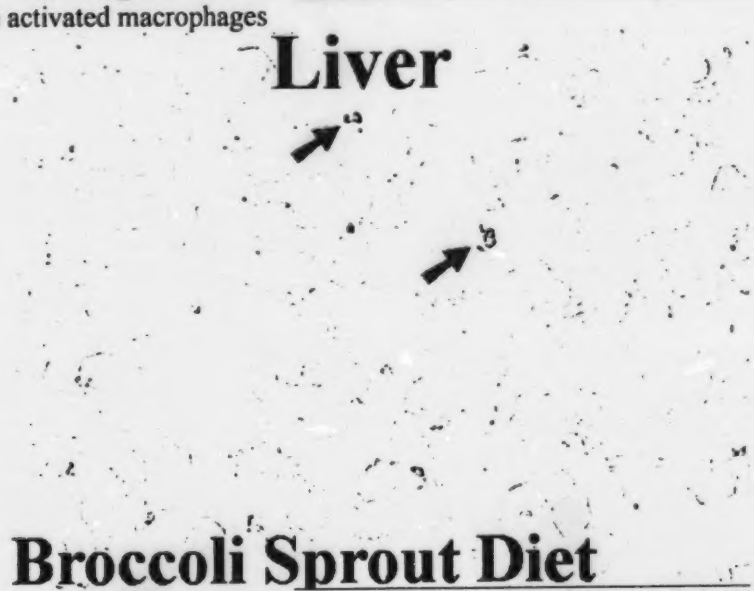


Figure 8



Control Diet

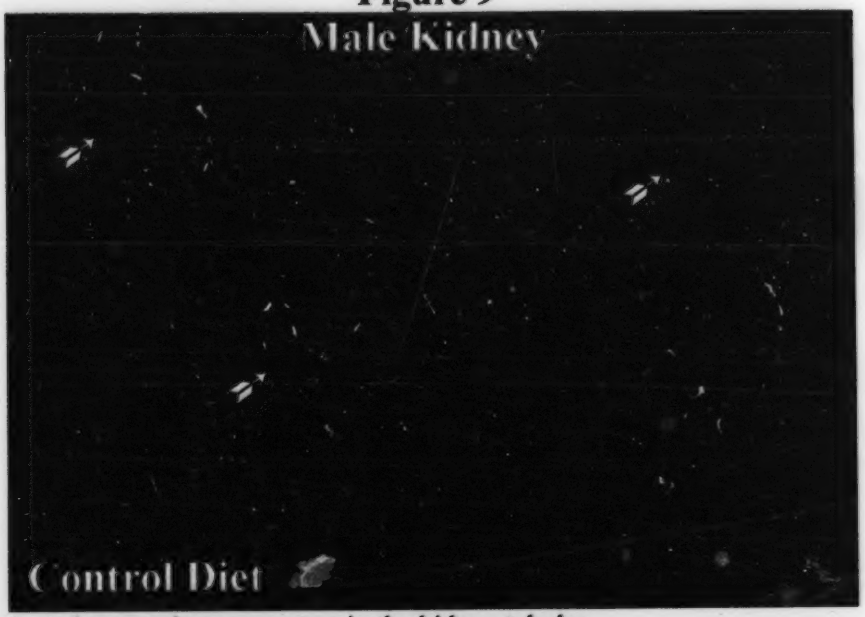
Arrows indicate activated macrophages



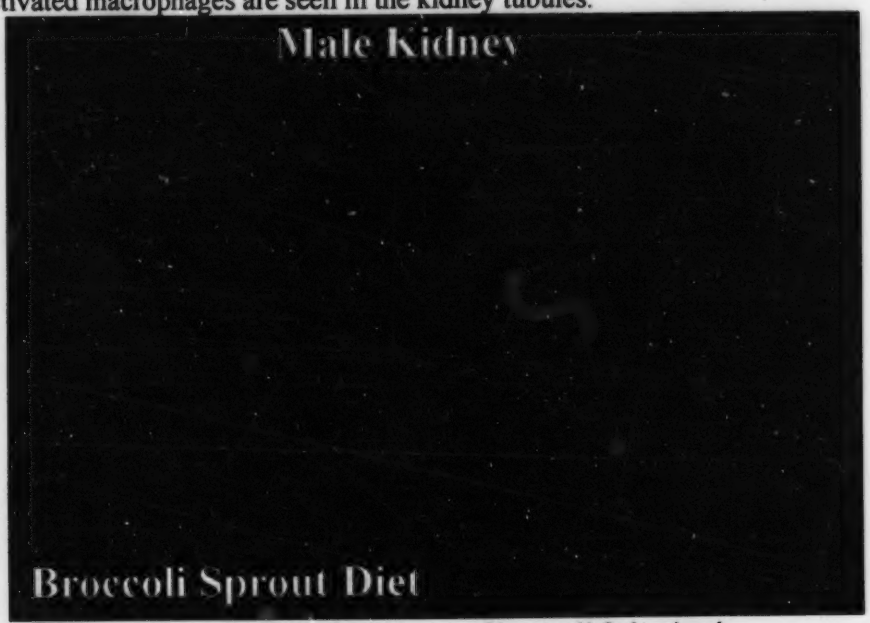
Broccoli Sprout Diet

There are far fewer activated macrophages in the livers of broccoli sprout fed animals.

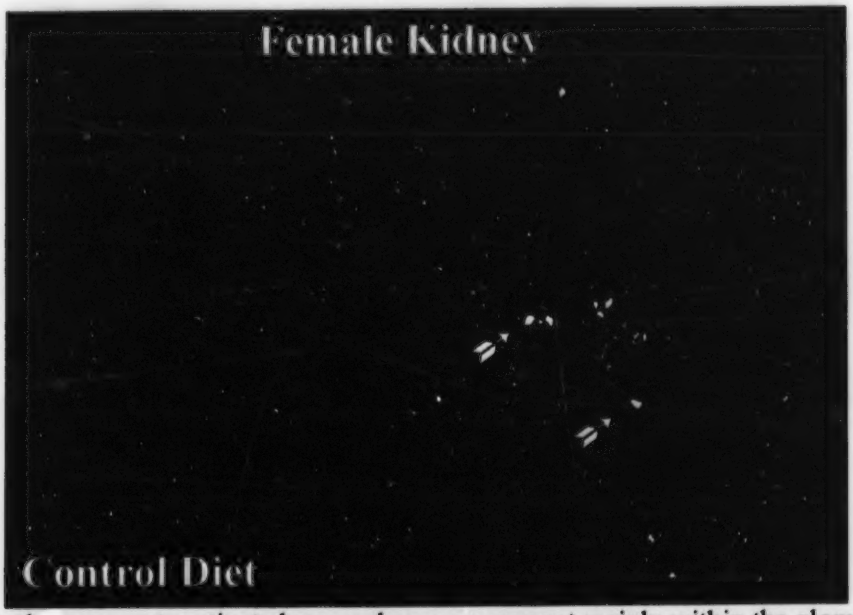
Figure 9



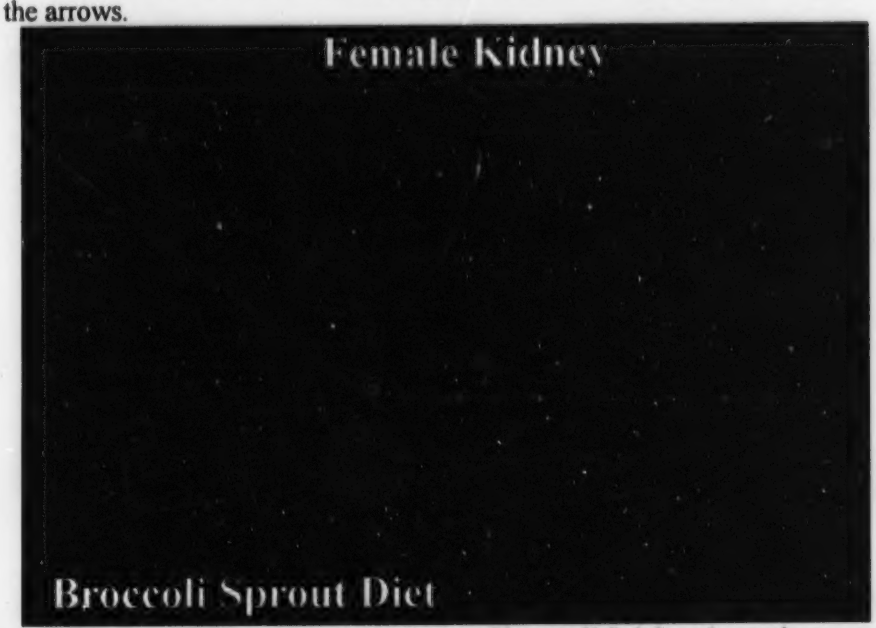
In males activated macrophages are seen in the kidney tubules.



Activated macrophages are not seen in the kidneys of broccoli fed animals.



In female stroke-prone rats activated macrophages are present mainly within the glomeruli as shown by the arrows.



Activated macrophages are not seen in the kidneys of broccoli fed female stroke-prone rats.

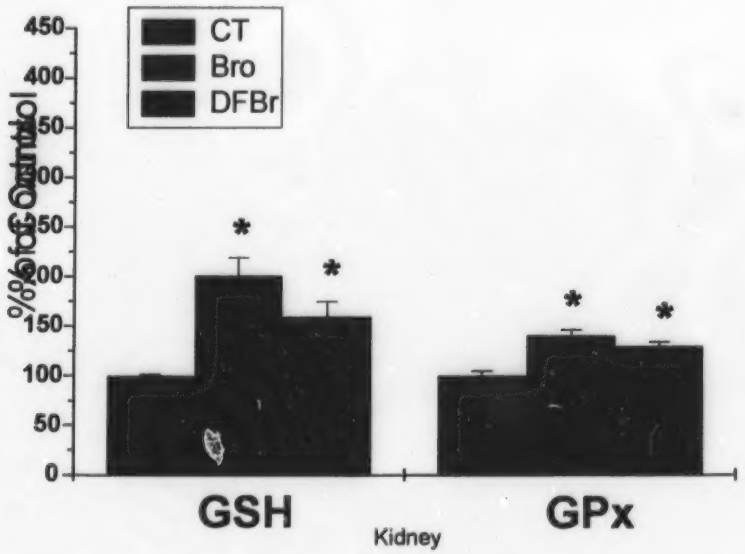
Figure 10

These figures demonstrate activated macrophages (brown staining structures) in the striatal part of the brain in stroke-prone male animals. It is clear that there are many more activated macrophages in the control diet fed animals than in the broccoli sprout fed animals.

Control Diet

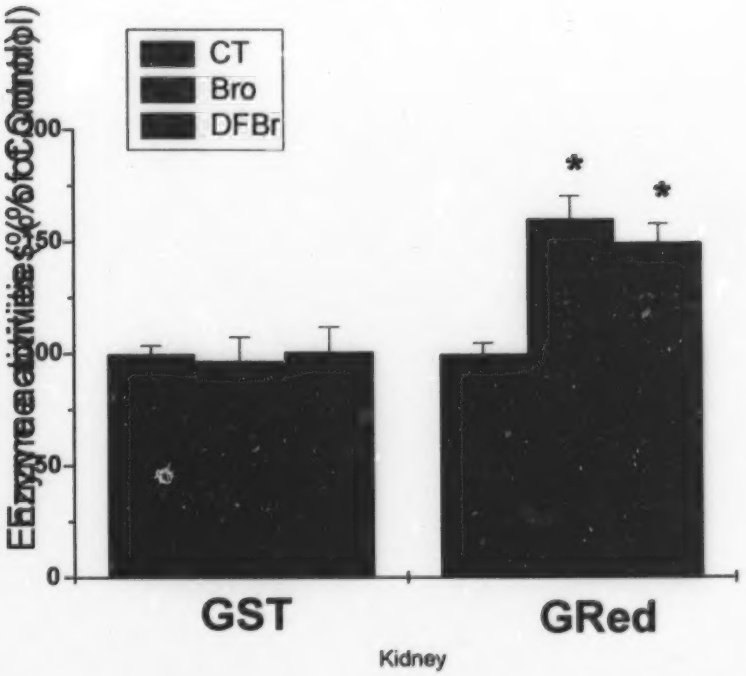
Broccoli Sprout Diet

Figure 11



CT = data from animals on control diets, Bro represents data from animals fed dried broccoli sprouts while DFBr represents data from animals fed broccoli sprouts that had been frozen, thawed and then dried. It is clear that animals fed with broccoli sprouts have a significantly higher content of glutathione (GSH) and significantly higher activities of glutathione peroxidase (GPx) in kidneys than do animals on the control diet. Glutathione peroxidase plays a very important role in scavenging peroxides, sources of strong oxidants, using glutathione as the electron donor.

Figure 12



Feeding animals broccoli sprouts also increased the glutathione reductase (GRed) activity but had no effect on the glutathione S-transferase (GST) activities of the kidneys. Glutathione reductase plays an an important role in the anti-oxidant defense system by reducing oxidized-glutathione.

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